GENOMIC SEQUENCING OF SINGLE CILIATE PROTOZOA CELLS FROM EQUINE FECAL MATERIAL

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Bachelor of Science in Pre-Veterinary Medicine & Animal Biosciences with Distinction

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ABSTRACT

The equine hindgut is home to a myriad of microbes including bacteria, archaea, fungi, and protozoa that allow for digestion and usage of a forage-based diet. This study sought to expand the current 18S database for equine gut protozoans by optimizing a ciliate-specific PCR protocol. Fresh equine fecal samples were collected and combined with 1X PBS to create a slurry solution. This slurry underwent filtration steps which included a series of five filters of decreasing size (200µm, 100µm, 70µm, 40µm, 10µm). Samples were observed under an inverted microscope and protozoans were manually single sorted. Cells were washed from debris and stored at -80°C in a 1-µL droplet in a PCR tube. DNA extraction optimization included two different series of freeze/thaw steps and a Chelex 100 mechanical disruption protocol. PCR amplification was optimized using ThermoFisher Scientific Phusion™ High-Fidelity DNA Polymerase and P-SSU54F or 82F and P-SSU1747R or EkyB primers (Ito et al., 2014, Sylvester et al., 2004). Custom designed inner primers, VegaF0721 and VegaR0821, were used in a nested primer approach for Sanger sequencing. Data from previously unsequenced species *Blepharoconus benbrooki* and *Blepharocorys valvata,* were obtained, as well as sequences from already sequenced species *Cochliatoxum periachtum,* and *Tripalmaria dogieli*. It was observed that the forward reads for each sequence were of high quality, and thus the forward primer proved to be more successful. It is recognized that the reverse primer produced poor reads and these sequences were not used within the study and the VegaR0821 inner primer needs to be

re-evaluated for effectiveness. A small equine dataset (n=45) was obtained from the Equine Microbiome Project (Berg et al., 2017) to determine the prevalence of the five species obtained from single sorting experiments in equine fecal samples. Results showed that the five single-sorted species were highly prevalent within the samples, validating the approach and need for an expansion of the 18S database. Neighbor-Joining trees revealed that the putative P46-*Blepharoconus benbrooki* and P42-*Blepharocorys valvata* samples did not cluster close to other species in their genus. It is to be noted that the Blepharocorythidae family has the possibility of being polyphyletic (Cedrola et al., 2021) and thus may account for the differences seen within the *Blepharocorys valvata* samples.

Chapter 1

LITERATURE REVIEW

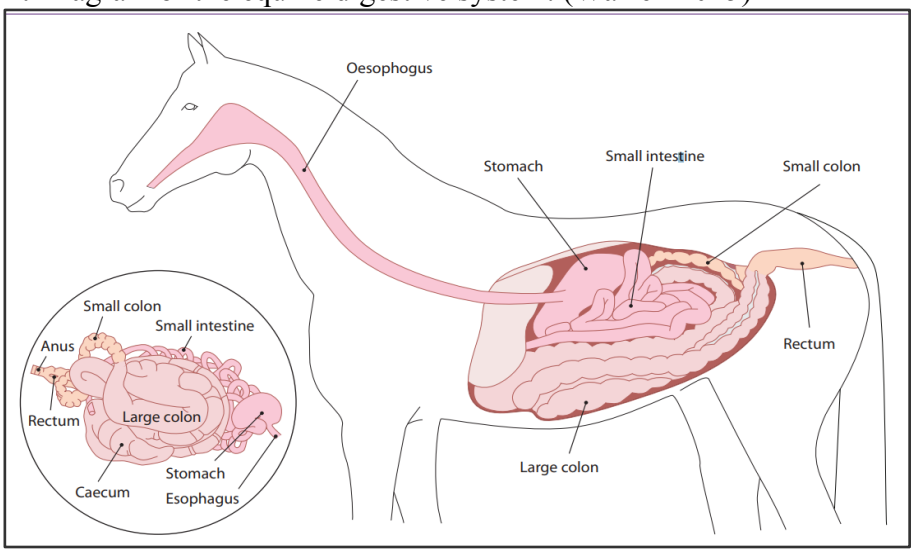
1.1 Equine Digestion

The equine's hindgut fermenting digestive system enables the obligate herbivorous animal to utilize and live off a forage-based diet. The fermenting digestive system is broken up into two regions, the foregut, and the hindgut. The foregut is much like the monogastric digestive system as it has one true stomach and small intestine. In the foregut, digestion is limited to the animal's own enzymes such as pepsin within the stomach. The feed passes through the stomach quite quickly relative to the rest of the digestive system (Dicks et al., 2014) and enters the small intestine, where enzymatic breakdown and absorption occur. In the small intestine, the acidic digesta is neutralized through bile secretion and fats are emulsified (Dicks et. al 2014). Soluble carbohydrates are hydrolyzed, and lactic acid, fatty acids, vitamins, and minerals are absorbed within the small intestine (Dicks et al., 2014).

Once the feed passes through the small intestine, it enters the hindgut region which begins with the caecum and colon. The hindgut is where the digestion of recalcitrant plant material occurs due to prolonged microbial fermentation (Warren 2015). Digesta will remain in this part of the digestive system for more than 60% of its entire retention time (Miyaji et al., 2008). The digesta is fermented by a specialized

microbial community that is housed in the hindgut, producing short-chain fatty acids that are essential for the horse's energy needs. Nutrients that were not degradable by endogenous enzymes are now able to be utilized and absorbed within the hindgut (Glatter et al., 2019). The digesta then reaches the small colon, where fecal balls are formed, and water is absorbed. Digesta moves into the rectum where it is defecated as fecal balls.

Figure 1: Diagram of the equine digestive system. (Warren 2015)



1.2 The Equine Microbiome

Microbes are found in all regions of the equine gut system. However, the equine microbiome can be separated into the above-mentioned regions - the foregut, and hindgut. It has been studied that the foregut microbiota is much more variable, due to the fast rate at which forage is traveling through this region, the acidity of the stomach, as well as the environmental microbes present within the ingested forage.

The hindgut, however, expresses a stable community across individual animals (Kauter et al., 2019).

Equids have the capability of surviving off a forage-based diet due to the complex and specialized microbial community housed in the hindgut. The hindgut microbial community contains bacteria, anaerobic fungi, protozoa, and archaea which all play a role in essential nutrient supplementation for the host (Kauter et al., 2019). When looking further into the community, it is seen that each microbial fraction and members are responsible for different metabolic niches. For example, *Ruminococcus albus* is known for plant wall degradation, while *Butyrivibrio spp.* is known for its cellulolytic and fibrolytic properties (Kauter et al., 2019). The community comes together to allow the host to access nutrients that it would otherwise not be able to use, providing it with 60-70% of its daily energy needs (Bełżecki et al., 2016).

1.3 Protozoans

Protozoa are unicellular eukaryotic organisms found in aquatic, terrestrial, and mammalian gut systems (Lynn, 2008). Most species of protozoa are free-living and vary in size and shape. Protozoans range in size from <10 μm to >300 μm (Lynn 2008). These organisms have a complex internal structure composed of organelles that are used to carry out various metabolic processes (Yaeger et al., 1996). There are both commensal and pathogenic protozoa species that fall under the Protozoa subkingdom. Protozoans divide either asexually, most commonly through binary fission, or sexually

through the production of gametes (Yaeger et al., 1996). The nutrition of all protozoans is similar in that they are all holozoic, requiring organic material (Yaeger et al., 1996). This diverse and ubiquitous group has a range of niches, and although it is possible to draw from research done on groups in other habitats, gut protozoans are relatively understudied.

1.4 Ciliated Gut Protozoa Research History

The predominant classes of protozoa found in the equine caecum and colon are Rhizopodosa, Mastigorphora, Ciliata, and Suctoria, the majority being ciliated protozoa (Julliand and Grimm, 2016). These ciliated protozoans were first described by Gruby and Delafond in 1843 within rumen samples. A large portion of the research history surrounding these gut protozoans is found in ruminant samples and there is limited research on equine protozoans. Many studies have relied and continue to rely on morphological identification (Newbold et al., 2015) which is a major limitation on the progression of molecular techniques.

Morphological descriptions and drawings of ciliated protozoa from different samples within the equine digestive system were described in Buisson (1923) and Hsiung (1930). Then in 1953, Adam described protozoa distribution within horses on varying diets. Later in 1983, Ike used non-invasive techniques to collect samples from horses of varying daily routines (Ike et al., 1983). This use of fecal samples expressed the ability to accurately depict the hindgut protozoa community through non-invasive

methods. Since the early 2000s, there has been a rise in studies focusing on equine protozoans, and with the advancement of technology, the ability to capture better quality images to identify species has become much easier (Cedrola et al., 2019, Göçmen et al., 2012, Gürelli et al., 2011, Gürelli et al., 2019, Strüder-Kypke et al., 2007).

Lynn highlighted protozoa diversity in a myriad of mammalian gut systems (Lynn 2008), and this led to the function of these microbes becoming a new investigative interest. Digestive functions of protozoans concerning cross-feeding relationships with methanogenic archaea, fiber degradation (Newbold et al., 2015), and microbial protein (Firkins and Mackie 2020, Hartinger et al., 2018) have been investigated and brought recognition to the importance of these microbes within the gut community. Although these papers focused on rumen-specific ciliate protozoa, many of these approaches could possibly be translated to equids.

In conjunction with function, the importance of inter-microbial relationships has also come to focus. Bacterial-protozoal relationships have been described through prey-predator interactions where specific bacterial groups are sought out for nutritive purposes. However, bacterial-protozoal relationships may also be commensal as the by-products of nitrogen fixation, acetogenesis, and methanogenesis serve as electron-sinks (Levy and Jami 2018, Ozutsumi et al., 2005). Protozoans have also been observed to house intra- and extra-cellular prokaryotic cells expressing a symbiotic relationship between these microbes (Levy and Jami 2018).

1.5 Equine Protozoa Reference Manual

The current gold standard for identifying equine protozoans is through morphological identification. Rumen specific gut protozoan guides are available (Dehority 2018, Dehority 2005, Lynn 2008) but no guides are currently updated for equine gut protozoans. Therefore, as a summer scholar in 2020, we constructed the Equine Protozoa Reference Manual (EPRM) containing 28 literature references and over 250 reference photos. The manual in its entirety consists of 116 species-level entries that allow researchers to easily identify protozoans microscope-side.

Species entries are organized into separate chapters based on organism size fraction (>200µm, >100µm, >70µm, >40µm, >10µm, <10 µm). Each protozoan species entry includes information describing its morphology and reference photo, mammalian host(s), geographical location, and NCBI sequence accession number. The EPRM does contain mammalian hosts other than the horse, but equine protozoans have been delegated their own section (Chapter 3: Equine Specific Protozoa). The dearth of NCBI sequence accession numbers for protozoal species in the EPRM shows the significance of the current study.

Figure 2: Example species entry within the Equine Protozoa Reference Manual

16. Triadinium caudatum

a. Size Range: L (50-105); W (36-85)
b. Size Mean: 72.88 x 55.16
c. Other fractions: 100µm, 40µm, 10µm
d. Reference: Hsiung, 1928 (page 100-101, fig 59-60)
e. Habitat: colon of horse (Hsiung), feces of horse (Gurelli 2019)
    i. Italy, South Africa, USA, Russia, Japan, Mexico, Taiwan, China
f. Morphology description:
    i. Body Shape: rigid, noncontractile, tail attached at posterior end
    ii. Ectoplasm and Endoplasm: ectoplasm is homogenous, endoplasm is granular and has food in it
    iii. Cuticle: covers the entire body, forms a knob at posterior end
    iv. Membranelles: anterior arch is short, dorsal arch is in posterior end
    v. Peristome: middle of ventral surface, surrounded by adoral membranelles
    vi. Esophagus: funnel-shaped, directs antero-dorsally
    vii. Cytopyge: posterior end, dorsal to caudal knob
    viii. Macronucleus: twisted, tail points dorsally, granular
    ix. Micronucleus: small, subspherical, lives on macronucleus tail
    x. Contractile Vacuole: one, anterior to macronucleus curve

g. Taxonomy
    i. Eukaryota, Sar, Alveolata, Ciliophora, Intramacronucleata, Litostomatea, Trichostomatia, Entodiniomorphida, Ditoxidae
h. Picture: #55C Hsiung 1928 (Plate IV), Ito 2014 (fig 21), Buisson 1923 (fig 21), Gurelli 2011 (fig 38)

1.6 Current 18S Work and Single Sorting Methods

The 18S rRNA gene has nine hypervariable regions, and like the 16S rRNA gene in prokaryotes, can be used as a biomarker for identification purposes (Ishaq and Wright 2014). The increase in interest to molecularly characterize the protozoans within the last 15 years (Lee et al., 2019, Ishaq and Wright 2014, Ito et al., 2014, Magnet et al., 2014, Mishra et al., 2020, Newbold et al., 2015, Skillman et al., 2006) has advanced the ciliated protozoa field immensely with the creation of ciliate-specific primers (Ishaq and Wright 2014, Sylvester et al., 2004) and has led to profiling the protozoal fractions more deeply within rumen and equid species (Kittelmann et al.,

2015, Mishra et al., 2020, Skillman et al., 2006). Protozoal discovery is typically done using morphological identification of novel protozoans to single sort and sequence individual protozoan species to improve molecular-based identification techniques (Ito et al., 2014, Lee et al., 2019). The development of ciliate-specific primers (Sylvester et al., 2004, Wright et al., 2014) to amplify the 18S gene has progressed this research in equine and rumen gut protozoans. Molecular techniques enable a myriad of possibilities including profiling, the discovery of novel protozoans, exploration of symbioses, evolutionary experiments, and more.

1.7 Objectives

This study addresses the limitation in the current database of full-length 18S sequences. The first objective was to single-sort morphologically identified, but molecularly ambiguous protozoa species to expand the 18S database. The second objective was to optimize PCR amplification conditions for the single-sorted equine protozoa species. The third objective was to validate the presence of these ambiguous species in 18S surveying of equine fecal samples.

Chapter 2

MATERIALS AND METHODS

2.1 Sample Collection

Fresh fecal samples were collected from eight horses after defecation. All but one was housed on the same farm, and all horses were located in the mid-Atlantic region, within 10 miles of the University of Delaware. All horses had free access to fresh pasture, as well as fed a mixed rations, and no animal had any underlying health conditions. A total of 116 samples were taken on 27 randomized days from 3/15/21 to 1/19/22, therefore samples were collected during all seasons and weather patterns. Protozoans were seen in all samples.

Samples were processed the day of collection and protozoa were single sorted within a few hours of sample collection. A fresh fecal ball was collected within two mins of defecation and was placed in a plastic cup containing 100 mL of warm 1X PBS solution. The sample was transported to the lab in a styrofoam box within an hour of collection.

2.2 Sample Preparation and Filtration

The solution containing the sample was mixed thoroughly by shaking the cup gently and filtered through a 1/16-inch nylon mesh strainer. The liquid filtrate was transferred to a 330μm stomacher bag. This step was done to remove any large fibrous debris. The sample then underwent filtration through a series of five filters of

decreasing size (200µm, 100µm, 70µm, 40µm, 10µm). These size fractions were chosen based on the diverse range in size of these organisms and having these separate fractions allowed lab members to choose specific size fractions to find specific protozoa species. The residue left on each filter was washed with 1X PBS into a falcon tube corresponding to filtrate size. Samples were stored at room temperature for up to five hours before processing.

2.3 Single Sorting and DNA Extraction

An aliquot of 100µL of filtered samples of each fraction size was transferred to a 6-well plate dish and diluted with 100µL-200µL 1X PBS. These samples were observed with an Olympus IX50 inverted microscope and individual protozoal species were identified by morphology using images and descriptive characteristic guidelines according to the Equine Protozoa Reference Manual (Vega et al., 2020). The ToupView microscope imaging software program (ToupTek ToupView 4.10 (http://www.touptek.com) accessed 12 November 2021) was used to capture microscopy images while single sorting individual protozoan cells.

Individual protozoa cells were transferred in a 1µL droplet to a clean well. The individual protozoa cell was washed with 1-µL of 1X PBS and transferred to a 1-µL droplet of clean 1X PBS two to three times to successfully clean the protozoan (Ito et al., 2014). Removing the cell from loose debris was essential in preventing contamination and incorrect DNA amplification. After the final PBS wash, the

protozoan was transferred in a 1µL droplet and stored at -20◦C in a PCR tube until it was further processed in one of the DNA extraction protocols.

Three published DNA extraction protocols were tested for the novel and single sorted protozoans. Protocol 1 was adapted from Hamilton et al., 2015 as follows: A 200µL 10% Chelex 100 solution is mixed with the 1µL sample, followed by a 20-minute incubation at 95^{o} C and a 15 s vortex at 14000 RCF. Protocol 2 was adapted from Ito et al., 2014 as follows: The PCR tube was frozen at −80 ◦C for five min and thawed at 60 ◦C for 30 s to facilitate cell breakage, this was repeated three times. Followed by incubation at 37◦C for 30 min, and 95◦C for five min. Protocol 3 was also adapted from Ito et al., 2014 as follows: The PCR tube was frozen at −80 ◦C overnight, followed by incubation at 37◦C for 15 min, and 95◦C for 2.5 min.

2.4 PCR Amplification and Gel Electrophoresis

Using ThermoFisher Scientific Phusion™ High-Fidelity DNA Polymerase, a 50µL reaction mastermix recipe of 35µL PCR H_2O, 10µL GC buffer, 1µL 10 nM dNTP, 0.5µL of 100µM 82F/P-SSU-54F primer, 0.5µL EkyB/P-SSU-1747R primer (Ito et al., 2014, Sylvester et al., 2004), 2.5µL template DNA, 0.5 µL Phusion polymerase, was created in this exact order. The PCR conditions used were adapted from Sylvester et al., 2004, adaption is seen in the initial denaturation temperature. The amplification conditions were 98°C for 4 min of initial denaturation; 34 cycles of

denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min; and a final extension at 72°C for 6 min.

Five µL of PCR product was combined with 1µL 6X dye and SYBR safe (ThermoFisher, Waltham, MA) and run in a 10% agarose gel electrophoresis. The gel was observed on an Axygen Gel Documentation System (Durham, North Carolina) under UV302 for bands at ≥1300 base pairs. Samples with bands were then tested for DNA quality using a Qubit 3.0 fluorometer (Wilmington, DE).

2.5 Primer Design

A classifier from Kittelmann et al., 2015 was adapted to include 11 more sequences, found in the EPRM, for a total of 179 ciliate gut protozoans which were then aligned in Mega-X (Mega-X 10.1.8 (https://www.megasoftware.net) accessed 15 April 2022) to find common motifs within the 1979bp amplified region by the P-SSU-54F/P-SSU-1747R primers (Sylvester et al., 2004), and 1878bp amplified region by the 82F/EkyB primers (Ito et al., 2014) of the 18S RNA gene that could act as inner primers for a nested-primer approach. These motifs were assessed for primer compatibility using the Primer3 online tool (Primer3 (https://www.primer3.org) accessed on 5 August 2021). The designed nested primers VegaF0721 (5'-AAACTGCNGAATGGCTCA-3') and VegaR0821 (5'-GGTGAACCTNTTTGGAC-3') were chosen. VegaF0721 had a GC content of 47.06% and VegaR0821 had a GC content of 50%. The annealing temperature found to be most compatible according to

the Tm ThermoFisher Calculator (Tm Calculator (thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/tm-calculator) accessed on 5 August 2021) was 54.9°C. These Vega inner primers amplify a 1656 bp region. The VegaF0721 primer is 60 bp away from the P-SSU-54F primer, and 32 bp away from the 82F primer. The VegaR0821 primer is 260bp away from the P-SSU-1747R primer, and 99 bp from the EkyB primer.

2.6 Sequencing

PCR product of P33, P42, P45, and P46 using outer primers P-SSU54F and P-SSU1747R (Sylvester et al., 2004) was sent to University of Delaware DNA Sequencing & Genotyping Center at the Delaware Biotechnology Institute (Newark, DE) for Sanger sequencing with newly created inner primers, VegaF0721 and VegaR0821. PCR product of P112 using outer primers 82F and EkyB (Ito et al., 2014) was sent to Genewiz (South Plainfield, New Jersey) for Sanger sequencing with newly created inner primers, VegaF0721 and VegaR0821.

2.7 Statistical Analysis

Sequences were analyzed using Geneious software (Geneious Prime 10.0.9 (https://www.geneious.com), accessed 20 April 2022), using its features to evaluate quality, create contigs and trim necessary regions. Geneious was also used to construct

and evaluate phylogenetic trees. The adapted classifier from Kittelmann et al., 2015 was aligned with the sequences gathered from this study and used to form two Neighbor-Joining phylogenetic trees with the Tamura-Nei genetic distance model and a support threshold of 50%, using *Giardia intestinalis* (Accession: XR_005248679) as an outgroup.

Qiime2 (Quantitative Insights Into Microbial Ecology, v 2020.8) was used to run the sequences collected against a database of horse fecal samples (n=45) to evaluate the presence of protozoan species collected within this study and to calculate average relative abundance. The workflow described in the "Moving Pictures" tutorial (https://docs.qiime2.org/2021.4/tutorials/moving-pictures/, accessed on 18 April 2022) was followed and taxonomic assignments were made against the Kittelmann et al., 2015 classifier trained to the P-SSU-316F/GIC758R primer set (Ishaq and Wright 2014).

Chapter 3

RESULTS

3.1 Sampling Results

The 1X PBS solution kept the protozoans in an osmotically favorable condition; however, no protozoan specimens were seen to be alive within any of the samples. The larger fractions (200 μm and 100 μm) were used most frequently within the experiment.

If samples were left overnight at room temperature, at 37◦C, or at 4◦C it was observed that in any of the stored conditions protozoa were not in optimal condition for morphological identification. Therefore, it was concluded that sample collection, processing, and single sorting must be achieved within the day.

3.2 Single Sorted Protozoa Figures

In total, 368 microscopy images were taken over the course of this experiment, capturing many equine gut protozoan species.

Figure 3: Two microscope images taken at 40X of *Tripalmaria dogieli* – sample P33. This protozoan seems to be dividing, seen by the cytoplasmic division.

Figure 4: Microscope image taken at 40X of *Blepharocorys valvata* – sample P42.

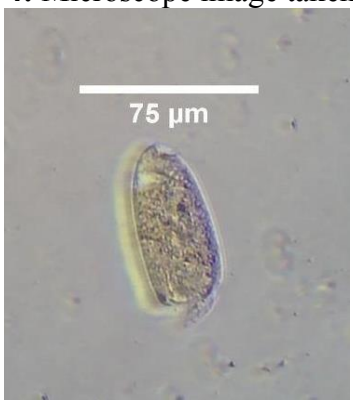


Figure 5: Microscope images taken at 40X of *Blepharocorys valvata* – sample P45.

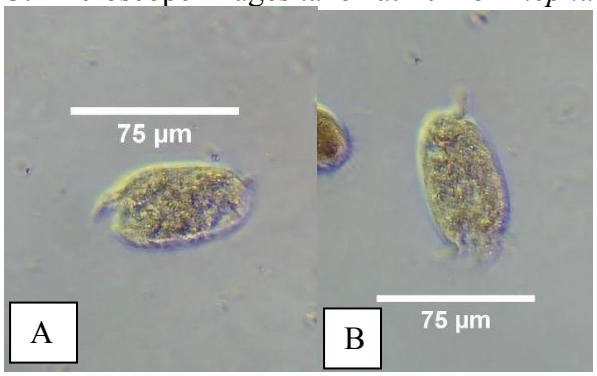


Figure 6: Microscope image taken at 40X of *Blepharoconus benbrooki* – sample P46.

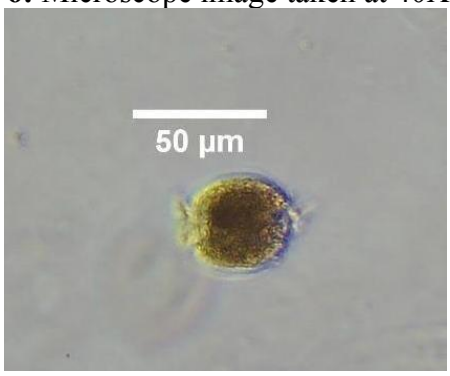
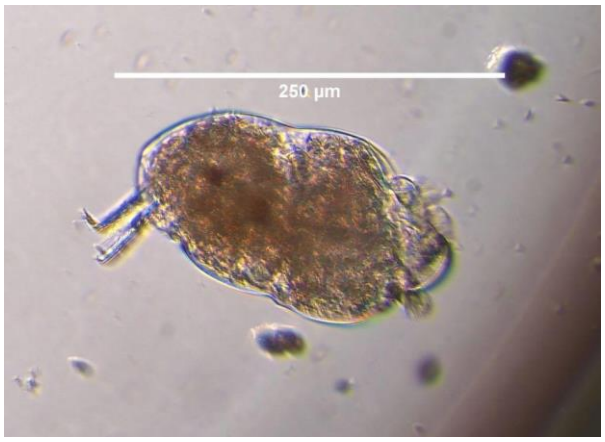


Figure 7: Microscope image taken at 40X of *Cochliatoxum periachtum* – sample P112.



3.3 DNA Extraction Protocol Results

It was determined that the three DNA extraction protocols within this study proved to be successful through the extraction and amplification of DNA from five protozoans (P33, P42, P45, P46 and P112). P33 and P42 underwent Protocol 2 (57.8 ng/uL and 62.2 ng/uL, respectively) P45 and P46 underwent Protocol 3 (52.6 ng/uL and 11.6 ng/uL respectively) and P112 underwent Protocol 1 (10.9 ng/uL). All

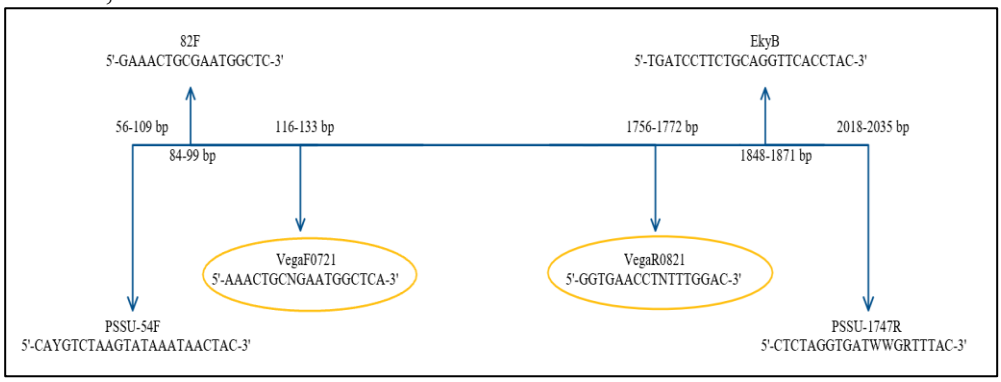
protocols extracted DNA successfully, seen through the Qubit analysis that was performed.

3.4 PCR Amplification Results

3.4.1 Primer Creation

Primers were created with the intention of finding a common motif among a majority of sequences within an adapted classifier of 179 sequences from Kittelmann et al., 2015. P-SSU-54F, P-SSU-1747R, 82F, and EkyB were used as markers for outer primers (Sylvester et al., 2004, Ito et al., 2014). It was found that the newly created forward primer, VegaF0721 5'-AAACTGCNGAATGGCTCA-3', covered 70% of sequences, and the reverse primer, VegaR0821 5'-GGTGAACCTNTTTGGAC-3', covered 82% of sequences.

Figure 8: Diagram depicting the newly created inner primers in reference to the outer P-SSU primers from Sylvester et al., 2004 and 82F and EkyB from Ito et al., 2014.



3.4.2 PCR Protocols

In total, six PCR protocols were tested (Table 1) (Sylvester et al., 2004, Ito et al., 2014, Ishaq and Wright 2014), and it was determined that the following protocol (Protocol 1) worked best and amplified five species sequences: 98°C for 4 min of initial denaturation; 34 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min; and a final extension at 72°C for 6 min.

Table 1: Expresses the six protocols that were investigated before optimizing 'Protocol 1' that was used within the current study.

	Total volume (µL)	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
Protocol 1*	**50 µL**	**98°C, 4 min**	**34X 94°C, 30 s**	**34X 55°C, 30 s**	**34X 72°C, 2 min**	**72°C, 6 min**
Protocol 2	50 µL	94°C, 4 min	34X 94°C, 30 s	34X 55°C, 30 s	34X 72°C, 2 min	72°C, 6 min
Protocol 3	20 µL	94°C, 4 min	30X 94°C, 30 s	30X 55°C, 30 s	30X 68.3°C, 2 min	68.3°C, 6 min
Protocol 4	50 µL	98°C, 30 s	34X 98°C, 5 s	34X 58.9°C, 30 s	34X 72°C, 1.5 min	72°C, 10 min
Protocol 5	25 µL	94°C, 4 min	29X 94°C, 30 s	29X 55°C, 30 s	29X 72°C, 1 min	72°C, 6 min
Protocol 6	50 µL	98°C, 30 s	34X 98°C, 5 s	34X 50°C, 30 s	34X 72°C, 2 min	72°C, 10 min

3.5 Sequence Analysis

It was observed that the reverse sequences for the five samples collected did not have high quality reads and resulted in lower quality scores when creating a contig with the forward reads. Therefore, only the forward reads were used in the sequence analysis portion of this study.

The forward read sequences were analyzed with an error probability limit of 0.05 and trimmed accordingly. The average number of trimmed bases was 160.6, with an average final length of 859.8 bases.

Table 2: Expresses the high quality %, number of trimmed bases, and the final sequence length of each of the forward reads of the sequences obtained in the study. Bases were trimmed with an error probability limit of 0.05.

Sample ID	Species Name	HQ%	# of Trimmed Base Pairs	Sequence Length (bp)
P33	*Tripalmaria dogieli*	67.8	155	887
P42	*Blepharocorys valvata*	6.3	210	842
P45	*Blepharocorys valvata*	31	198	849
P46	*Blepharoconus benbrooki*	68.2	189	884
P112	*Cochliatoxum periachtum*	73.5	51	837

3.6 Statistical Analysis

Figures 9 and 10 are Neighbor-Joining trees that express the species obtained in this study and the clustering seen with sequences from the adapted Kittelmann et al., 2015 classifier.

Figure 9: Neighbor-Joining Tree expresses the branching of species obtained in this study, P33-*Tripalmaria dogieli* P42-*Blepharocorys valvata* P46-*Blepharoconus benbrooki* and P112-*Cochliatoxum periachtum,* with 32 species from the created classifier. *Giardia intestinalis* acts as the outgroup.

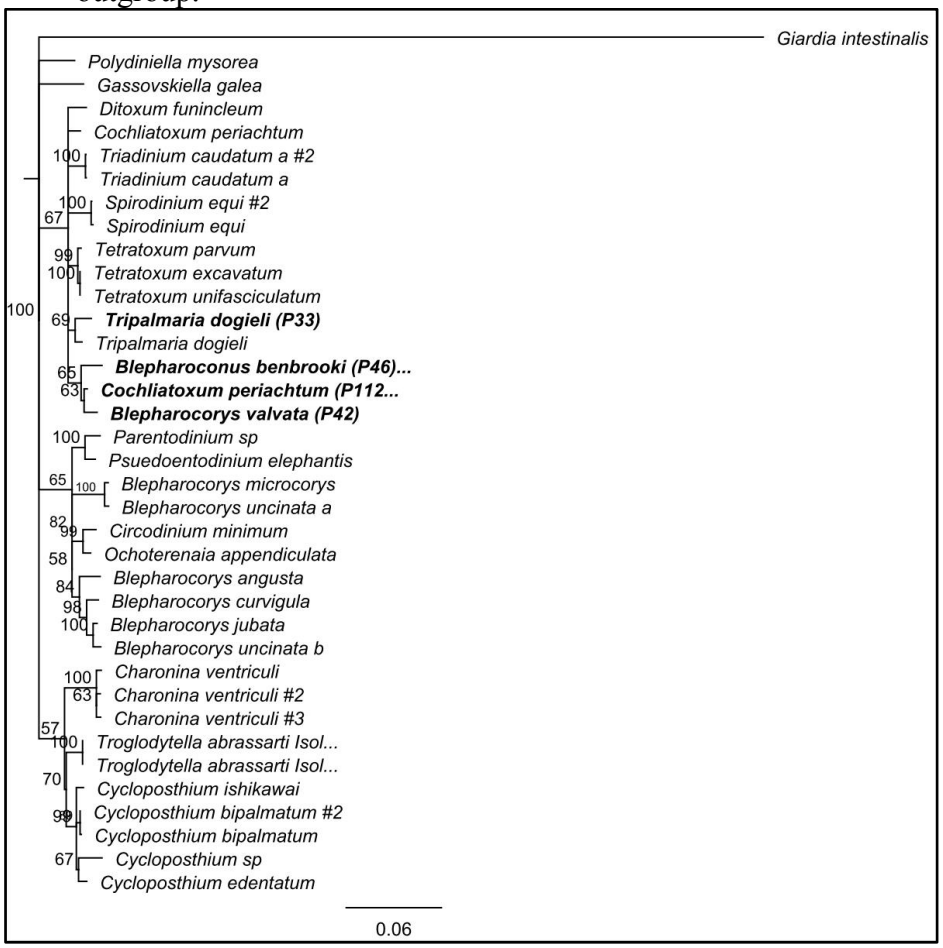


Figure 10: Neighbor-Joining Tree expresses the branching of one species obtained in this study, P45-*Blepharocorys valvata,* with 31 species from the created classifier. *Giardia intestinalis* acts as the outgroup.

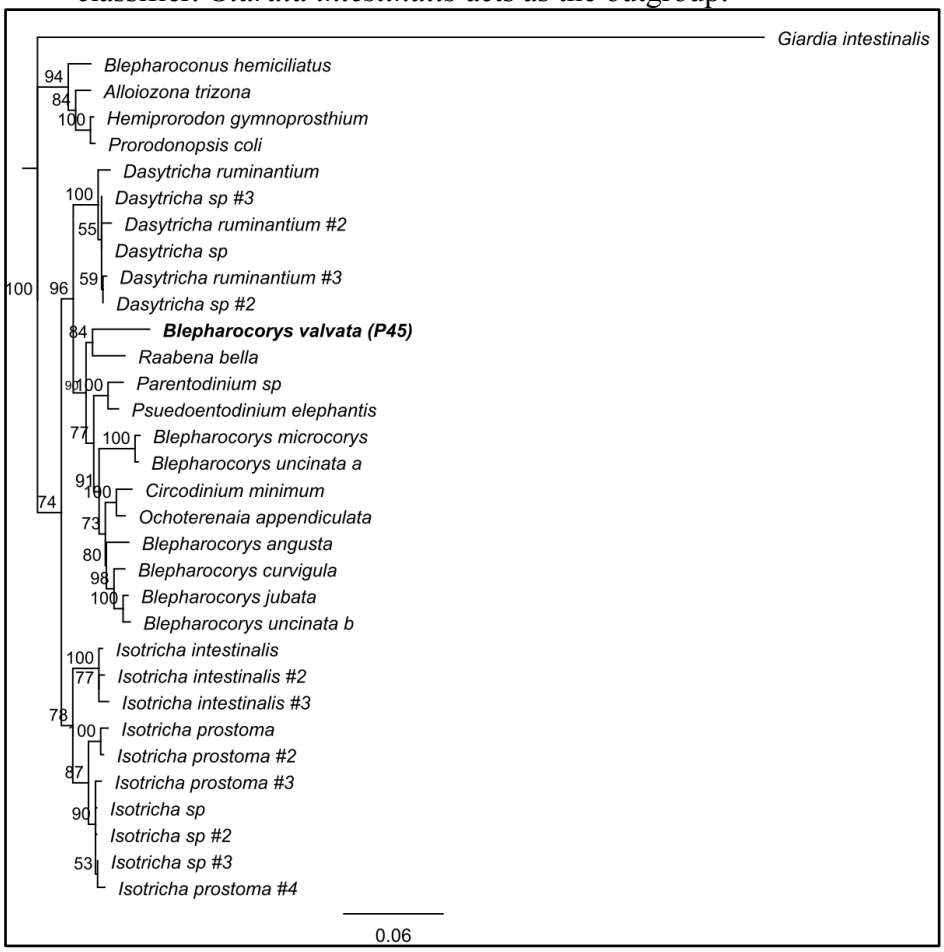
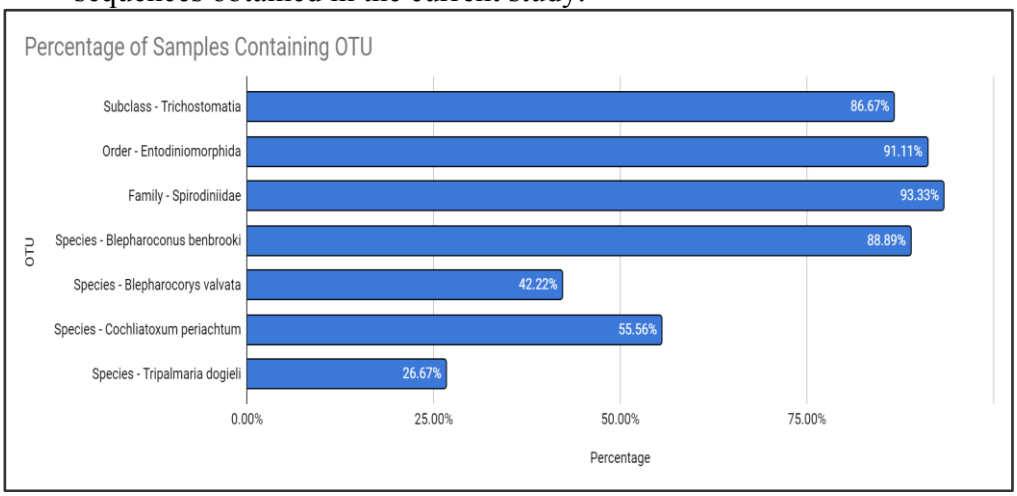


Figure 11 shows that *Blepharoconus benbrooki* was seen in 88.89% of 45 samples it was run against, *Blepharocorys valvata* 42.22%, *Cochliatoxum periachtum* 55.56%, and *Tripalmaria dogieli* 26.67%. Average relative abundance for each species was 14.8% ± 13.9, 0.64% ± 1.25, 2.52% ± 4.0, and 0.35% ± 1.24, respectively.

Figure 11: Percentage of samples within a database of 45 horses that contained the sequences obtained in the current study.



Chapter 4

DISCUSSION

Protozoans make up as much as 50% of the equine microbial biomass due to their large size (Bełżecki et al., 2016). However, within the equid, these eukaryotic organisms are not well studied. Morphological identification has remained the gold standard (Newbold et al., 2015), but a shift towards molecular procedures is now being seen. This shift has been a gradual process, and this current study expresses many reasons behind the slow progression of this field.

4.1 Sampling Methods

Using fecal material as a representative for the hindgut (Ike et al., 1983) has made sampling methods easier and less invasive. Given the ease of obtaining fresh fecal samples, the process of sampling has not contributed to the overall lack of progress toward molecular methods. Once these samples have been processed, however, the single sorting and morphological identification procedures begin and can be laborious and time-consuming since single sorting and identifying differences in protozoan morphological characteristics is a learning curve and is a skill that is acquired over time. The Equine Protozoa Reference Manual developed in the Biddle lab proved to be a valuable guide for microscope-side identification of many species.

4.2 Single Sorting Methods

Upon microscopy, it was observed that all samples contained protozoans. It was not surprising that the organisms were not alive upon viewing despite the osmotically favorable conditions they were kept in. Ciliate protozoans are strict anaerobes (Park and Yu 2018) and the method of acquiring fecal samples did not allow for an anaerobic environment, as would be needed for culture-based experiments in the future. The current study, however, did not need the cells to be alive, but instead morphologically intact for accurate identification. It was concluded that the 200 μm and 100 μm fractions were used most frequently within the experiment. It was found that the small protozoa often attached themselves to debris and were filtered out in the larger fractions along with the larger species, making these filter fractions the most useful in attaining a wide variety of species.

4.3 DNA Extraction

Lysis of the protozoa cells and amplification of the desired 18S region proved to be the hardest aspects of this experiment. Protozoans did not amplify equally on one single protocol demonstrating that each novel protozoan required a particular DNA extraction protocol which may be due to their size. Lysing particular protozoa species was more difficult than others. Smaller species such as *Blepharoconus benbrooki* and *Allantosoma intestinalis* broke very easily, while larger organisms such as *Cochliatoxum periachtum* and *Tripalmaria dogieli* were much hardier and resistant to

breakage. Studies such as that of Ito et al., 2014 observed larger protozoa cells which required tougher lysis protocols. After realizing the fragility of some species and the hardiness of others, the protocols were amended and optimized for species-specific cell lysis.

Three approaches were taken to try to lyse the cells efficiently. These protocols included two different freeze/thaw methods adapted from Ito et al., 2014 and a mechanical disruption method via Chelex 100 which was taken from Hamilton et al., 2015. All three protocols proved to be effective, as the sequences obtained in the current study had undergone one of the three methods to break open the cells and access the DNA. DNA extraction Protocol 1 with Chelex 100 worked best for larger protozoa species including *Cochliatoxum periachtum*. It was observed that Protocol 2 which consisted of three freeze/thaw cycles worked best for medium to large sized protozoa including *Tripalmaria dogieli* and *Blepharocorys valvata.* Lastly, Protocol 3 with the overnight freeze was best fit for medium to smaller sized species including *Blepharocorys valvata* and *Blepharoconus benbrooki*.

4.4 PCR Conditions

After ample modification of many PCR protocols (Ito et al., 2014, Ishaq and Wright 2014, Kim and Min 2009, Lee et al., 2019, Skillman et al., 2006, Sylvester et al., 2004), Protocol 1 as seen in Table 1 proved to be the most successful. All five species sequenced within this study underwent this PCR protocol. The 98°C initial

denaturation was recommended within the ThermoFisher Scientific Phusion™ High-Fidelity DNA Polymerase user manual. The 94°C denaturation, 55°C annealing, and 72°C extension are common in ciliate protozoal PCR amplification and these separate temperatures are seen across many experiments (Ito et al., 2014, Ishaq and Wright 2014, Kim and Min 2009, Lee et al., 2019, Skillman et al., 2006, Sylvester et al., 2004).

4.5 Nested Primer Approach

The use of a nested primer approach for 18S work has been developing in recent years (Hamilton et al., 2015, Hawash et al., 2014, Kittelmann et al., 2015, Lee et al., 2019). Using the primers from Sylvester et al., 2004 and Ito et al., 2014, the current study aimed to create successful ciliate-specific inner primers, VegaF0721 and VegaR0821. The nested approach is known to be effective at preventing incorrect or false amplification product (Lee et al., 2019) and was therefore used in the current study as a tool to optimize the PCR protocol. In situations such as the current study where there may not be sufficient initial DNA within the cell, the outer primer set allows for amplification of the target region and the inner primers then anneal to the internal region of the outer pair (Lee et al., 2019). This optimization through a nested primer approach was seen through gel electrophoresis quantification. When a nested approach was introduced into the protocol, clearer and brighter bands formed in the expected base pair region.

The forward primer, VegaF0721 proved to be more successful in proper amplification of equine protozoan DNA than the reverse VegaR0821 primer. The forward sequences all had higher quality reads, and the use of reverse reads to create a contig resulted in lower quality sequences. For this study, only the forward reads were used, and it is recognized that the reverse primer VegaR0821 should be re-evaluated for effectiveness in amplifying equine specific protozoan DNA.

4.6 Phylogenetic Analysis

The Neighbor-Joining trees created using the sequences from this study and those found in the adapted Kittelmann et al., 2015 classifier demonstrated interesting clustering patterns. In Figure 10, it is seen that P33-*Tripalmaria dogieli* aligned with its known species sequence, which was expected. P112-*Cochliatoxum periachtum* was further from its known species sequence, but still had a bootstrap value of 67, expressing some similarity between the sequences. This lower bootstrap value may be indicative of a shorter attained read within this study. Previously unsequenced species samples, P42-*Blepharocorys valvata* and P46-*Blepharoconus benbrooki* did not cluster close to other species in their respective genera. It has been noted that the Blepharocorythidae family may be polyphyletic, possibly due to human bias and difficulty in past morphological identification of these organisms (Cedrola et al., 2021). This unexpected clustering of these unsequenced species thus can elucidate a genetic divergence from the other species that share the same genus. This finding may

also express a phylogenetic difference of species found specifically in equine samples. Figure 11, however, expresses a separate *Blepharocorys valvata* sample, P45, that does have a closer clustering to the other Blepharocorys species, with a bootstrap value of 96. This begs the question of if two separate organisms of the same protozoan species can have multiple copies of the 18S region of interest that are genetically dissimilar. Human error may also be a possible factor in the discrepancy of the *Blepharocorys valvata* samples. It is possible that P42 may have been misidentified as morphological identification can be a difficult task. Contamination of the sample with a different protozoan species can also be another factor that would affect the attained sequencing information for this sample.

4.7 Single-Sorted Species Prevalence

Figure 9 expresses the percentage of samples within a database of 45 horses that contained the sequences obtained in the current study. The five protozoans that were collected and sequenced were very prevalent within the samples. *Blepharoconus benbrooki* and *Blepharocorys valvata*, previously unsequenced protozoan species, show a high percentage of presence, 88.89% and 42.22%, respectively, with average relative abundances of 14.8% ± 13.9 and 0.64% ± 1.2, respectively. The large abundance of previously unsequenced *Blepharoconus benbrooki* expresses the need for an expansion of 18S work to better profile the equine ciliate community. Lessons

and techniques learned from this work can be expanded to other eukaryotes found in the gastrointestinal system.

Chapter 5

CONCLUSION

This study successfully single-sorted and sequenced two previously unsequenced equine protozoan species, *Blepharocorys valvata* and *Blepharoconus benbrooki*. The addition of two species to the 18S database allows for a more accurate representation of the ciliate gut community.

A PCR protocol was optimized for four different ciliate species. These PCR conditions and its methodology can be used in future studies to amplify other morphologically identified but genetically ambiguous species.

The current study expresses the use of unique DNA extraction protocols for specific protozoan species, which has not been previously studied. This specificity may be the future of ciliate 18S sequencing and may expand the 18S database and allow for advancement of molecular work. The deficiency in a broad 18S database does not allow for accurate profiling of the eukaryotic community. With techniques and methods from this current study, 18S work can progress and molecular methods can be solidified as easy and accurate identification tools.

The use of a nested primer approach with the creation of inner primers proved to be a valuable technique for 18S work. Although VegaR0821 did not prove to be successful, VegaF0721 amplified high quality reads, and this can be a stepping-stone towards future optimization of ciliate-specific inner primers.

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Appendix A

Sequence Reads

Species	Trimmed Forward Sequence Read (5’ to 3’)
(P33) *Tripalmaria dogieli*	5’- TGTAGAAAACTAGAGCTAATACATGCCATAACCGCAAGG TTGTATTTATTAGATATTCCAAATCGGTGAATCATAATAA CTTCGCAAATCTCGTTTTTGACGAGATAAATCATTCAAGT TTCTGCCCTATCATGCTTTCGATGGTAGTGTATTGGACTA CCATGGCTTTCACGGGTAACAGGGAATTAGGGTTCGATTC TGGAGAAGGAGCCTGAGAAACGGCTACTACATCTACGGA AGGCAGCAGGCGCGTAAATTACCCAATCCTGACTCAGGG AGGTGGTGACAAGATATAACAACGCGATTACTATCGCGA TTGTAGTGAGGGTATTCTAAACCGAACCACTAGTACGATT AGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAG CTCTAATAGCGTATATTAAAGTTGCTGCAGTTAAAAGGCT CGTAGTTGGATTTCAAGGATTGTAAACCCTTACGGGAATA CATCCTACTAGTCATTGACTGTTACTGTGAGAAAATTAGA GTGTTTCAAGCAGGCTTTTGCAAGAATACATTAGCATGGA ATAACGAATGTATTTAGAATCTTGGTTAATTCTAAATTAC GATTAATAGAGACAGTTGGGGGCATTAGTATTTAATAGTC AGAGGTGAAATTCTTGGATTTATTAAAGACTAACGTATGC GAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGGACG AAAGATAGGGGATCAAAGACAATCAGACACTGTCGTAGT CCTATCTATAAACTATGCCGACTAGGGATTGGAGTGGGA ATACACCATTTTCAGTACCTTATGAGAAATCAAAGTCTTT GGGTTCTGGGGGGGGAGTATGGTCGCAAGGGCTGAAACT TAAAGAAATTGACGGA-3’
(P42) *Blepharocorys valvata*	5’- AACTAGAGCTAATACATGCCATAACCGCAAGGKTGTATT TATTAKATATTCCAAATCGGTGAATCATAATAACTTCKCA AATCTCGTTTTTGACGARATAAATCATTCAAGTTTCTGCC CTATCATGCTTTCGATGGTAGTGTATTGGACTACCATGGC TCTCACGGGTAACAGGGAATTAGGGTTCGATTCTGGAGA AGGAGCCTGAKAAACGGCTACTACATCTACKGAAGGCAG CAGGCGCGTAAATTACCCMATCCTGACTCAKGGAGGTGG TGACRAGATATAACAACGCSATTAAAATCGTGATTGTAGT GAGGGTATTCTAAACCGAACCACTAGTACGATTAKAGGG CAAGTCTGGTGCCRSCAGCCGCGGTAATTCCAGCTCTAAT AGCGTATATTAAAGTTGCTGCAGTTAAAAGGCTCGTAGTT GGATTTCAAGGCATGTAAACTCCTCTCGGAGAATACATCC TACTAGTCATTGACTGTTACTGTGAGAAAATTAGAGTGTT TCAAGCAGGCTTTTGCAAGAATACATTAGCATGGAATAA CGAATGTATTTAGAATCTTGGTTAATTCTAAATTACGATT AATAGAGACAGTTGGGGGCATTAGTATTTAATTGTCAGA GGTGAAATTCTTGGATTTGTTAAAGACTAACGTATGCGAA AGCATTTGCCAAGGATGTTTTCATTAATCAAGGACGAAA

	GATAGGGGATCAAAGACAATCAGACACTGTCGTAGTCCT ATCTATAAACTATGCCGACTATGGATTGGAATGGGTATTA CACCATTTTCAGTACCTTATGAGAAATCAAAGTCTTTGGG TTCTGGGGGGG-3’
(P45) *Blepharocorys valvata*	5’- AACTGTAGAAACTAGAGCTAATACATGCCATAACCGTAA GGTTGTATTTATTAGATATACTAAATAAGGTGAATCATAA TAACTTCGCAAATCTCAACGTCAGTTGAGATAAATCATTC AAGTTTCTGCCCTATCATGCTTTCGATGGTAGTGTATTGG ACTACCATGGCTTTCACGGGTAACAGGGAATTAGGGTTC GATTCTGGAGAAGGAGCCTGAGAAACGGCTACTACATCT ACGGAAGGCAGCAGGCGCGTAAATTACCCAATCCTGACT CAGGGAGGTGGTGACAAGATATAACAACGCGATTAACTT CGTGATTGTAGTGAGGGTATTCTAAACCGAACCGCTAGTA CGATTAGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAAT TCCAGCTCTAATAGCGTATATTAAAGTTGCTGCAGTTAAA AAGCTCGTAGTTGGATTTCAAGAAGTTTTTACATGTTTCT GCATGTAAAGCTTCTACTAGTCCTTGACTGTTACTGTGAG AAAATTAGAGTGTTTCAAGCAGGCTTTTGCAAGAATACAT TAGCATGGAATAACGAATGTGTTTAGAATCTTGGTTAATT CTAGATTACGATTAATAGAGACAGTTGGGGGCATTAGTA TTTAATTGTCAGAGGTGAAATTCTTGGATTTTTTAAAGAC TAACGTATGCGAAAGCATTTGCCAAGGATGTTTTCATTAA TCAAGAACGAAAGATAGGGGATCAAAGACAATCAGATAC TGTCGTAGTCCTATCTATAAACTATGCCGACTAGGGATTG GAGTGGTAGCATAGCACTTCAGTACCTTATGAGAAATCA AAGTCTTTGGGGTTCTGG-3’
(P46) *Blepharoconus benbrooki*	5’- TGTAGAAAACTAGAGCTAATACATGCCGTAACCGCAAGG TTGTATTTATTAGATATTCCAAATTGGTGAATCATAATAA CTTCGCAAATCTCGTTTTTGACGAGATAAATCATTCAAGT TTCTGCCCTATCATGCTTTCGATGGTAGTGTATTGGACTA CCATGGCTCTCACGGGTAACAGGGAATTAGGGTTCGATTC TGGAGAAGGAGCCTGAGAAACGGCTACTACATCTACGGA AGGCAGCAGGCGCGTAAATTACCCAATCCTGACTCAGGG AGGTGGTGACAAGATATAACAACGCGATTAAAATCGTGA TTGTAGTGAGGGTATTCTAAACCGAACCACTAGTACGATT AGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAG CTCTAATAGCGTATATTAAAGTTGCTGCAGTTAAAAGGCT CGTAGTTGGATTTCAAGGCATGTAAACTCTCCGGAGTTTG CATCCTACTAGTCATTGACTGTTACTGTGAGAAAATTAGA GTGTTTCAAGCAGGCTTTTGCAAGAATACATTAGCATGGA ATAACGAATGTATTTAGAATCTTGGTTAATTCTAAATTAC GATTAATAGAGACAGTTGGGGGCATTAGTATTTAATTGTC AGAGGTGAAATTCTTGGATTTGTTAAAGACTAACGTATGC GAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGGACG AAAGATAGGGGATCAAAGACAATCAGACACTGTCGTAGT CCTATCTATAAACTATGCCGACTAGGGATTGGAATGGGA ATAAACCATTTTCAGTACCTTATGAGAAATCAAAGTCTTT

	TGGGTTCTGGGGGGGGAGTATGGTCGCAAGGCTGAAACT TTAAAGAAATTTG-3’
(P112) *Cochliatoxum perichatum*	5’- AGCTNATACATGCCATAACCGCAAGGTTGTATTTATTAGA TATTCCAAATCGGTGAATCATAATAACTTCGCAAATCTCG TTTTTGACGAGATAAATCATTCAAGTTTCTGCCCTATCAT GCTTTCGATGGTAGTGTATTGGACTACCATGGCTCTCACG GGTAACAGGGAATTAGGGTTCGATTCTGGAGAAGGAGCC TGAGAAACGGCTACTACATCTACGGAAGGCAGCAGGCGC GTAAATTACCCAATCCTGACTCAGGGAGGTGGTGACAAG ATATAACAACGCGATTAAAATCGTGATTGTAGTGAGGGT ATTCTAAACCGAACCACTAGTACGATTAGAGGGCAAGTC TGGTGCCAGCAGCCGCGGTAATTCCAGCTCTAATAGCGTA TATTAAAGTTGCTGCAGTTAAAAGGCTCGTAGTTGGATTT CAAGGCATGTAAACTCCTCTCGGAGAATACATCCTACTAG TCATTGACTGTTACTGTGAGAAAATTAGAGTGTTTCAAGC AGGCTTTTGCAAGAATACATTAGCATGGAATAACGAATG TATTTAGAATCTTGGTTAATTCTAAATTACGATTAATAGA GACAGTTGGGGGCATTAGTATTTAATTGTCAGAGGTGAA ATTCTTGGATTTGTTAAAGACTAACGTATGCGAAAGCATT TGCCAAGGATGTTTTCATTAATCAAGGACGAAAGATAGG GGATCAAAGACAATCAGACACTGTCGTAGTCCTATCTATA AACTATGCCGACTNGGGATTGGAANTGGTATTACACCATT TCAGTACCTTATGAGAAATCAAAGTCTTTGGGTTCTGGGG GGAGT-3’

Supplemental 1: Table expressing the species obtained in this study with their respective trimmed forward reads.

Appendix B

Timeline

The start of this project began in June 2020 with the research and investigation of equine gut protozoans. After the assembly and completion of the Equine Protozoa Reference Manual in August 2020, single sorting protocols were explored. It was determined that sampling with 1X PBS and filtering with five size fractions of decreasing size was most optimal. The skill of single sorting individual protozoa cells took weeks to acquire.

In September 2020 after single sorting practice was well under way, DNA extraction and PCR protocols came into focus. Ito et al., 2014 was the first paper that was investigated for DNA extraction and PCR methods that could be applied to the current study. The original freeze/thaw protocol from Ito et al., 2014 was used in conjunction with a lysis buffer created from C1 and Powerbead contained in the Qiagen DNA Extraction Kit. Thermofisher Scientific DreamTaq Green PCR Mastermix was the first polymerase that was used for the initial start to PCR amplification. After multiple failed attempts at amplifying DNA, in November 2020 a new incubation step was introduced which is seen in the current protocols.

Months of trial and error passed until a new polymerase, ThermoFisher Scientific Phusion™ High-Fidelity DNA Polymerase, was tested in March 2021. In June 2021 sample P33 was collected, and in July samples P42, P45 and P46 were collected. At this time, a nested primer approach was investigated, and in July 2021 P-SSU primers from Sylvester et al., 2004 were used as outside primers. Inner primer Vega0721 was created, and Vega0821 followed in early August.

PCR conditions underwent testing in August 2021 with the creation of the current ‘Protocol 1’ PCR protocol that was used in the current study. During this month, samples P33, P42, P45, and P46 amplified successfully using P-SSU54F and P-SSU1747R primers (Sylvester et al., 2004) and the PCR conditions found in Table 1 under ‘Protocol 1’. These samples were all sent to University of Delaware DNA Sequencing & Genotyping Center at the Delaware Biotechnology Institute (Newark, DE) for Sanger Sequencing. From August until October more investigation of PCR conditions proved that Protocol 1 was most successful out of all the previous protocols. In November sample P112 was collected and a Chelex protocol from Hamilton et al., 2015 was used.

In January 2022 sample P112 amplified successfully using P-SSU54F and P-SSU1747R primers (Sylvester et al., 2004) and the PCR conditions found in Table 1 under ‘Protocol 1’. This sample was sent to Genewiz (South Plainfield) for Sanger sequencing in February 2022.